PATENT
Attorney Docket No.: 20695C-008700US
Client Reference No. PU-279.00

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of:

Varadi *et al.*

Application No.: 10/816,099

Filed: March 31, 2004

For: KITS FOR MEASURING THROMBIN GENERATION

Customer No.: 44183

Confirmation No. 9454

Examiner: Rosanne Kosson

Technology Center/Art Unit: 1653

DECLARATION OF DR. PETER TURECEK UNDER 37 C.F.R. § 1.132

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

1. I, Peter Turecek, being duly warned that willful false statements and the like are punishable by fine or imprisonment or both (18 U.S.C. § 1001), and may jeopardize the validity of the patent application or any patent issuing thereon, state and declare as follows:

2. All statements herein made of my own knowledge are true, and statements made on information or belief are believed to be true and correct.

3. I am a named inventor on the above-referenced patent application and I previously submitted a Declaration under 37 C.F.R. § 1.132 in support of the patentability of this application over various references cited in an obviousness rejection. The Declaration presented results from experiments demonstrating that (1) the addition of $CaCl_2$ to a fluorescent substrate in an aqueous solution leads to formation of a precipitate; and (2) dissolution of the fluorescent

substrate in an aqueous solution leads to the formation of a fine precipitate. It is my understanding that the obviousness rejection has been maintained.

4. The Examiner notes that concentrations, molar ratios and temperatures, which are parameters that influence solubility, are not provided in the previous Declaration and therefore concludes that the Declaration does not provided sufficient data to show that the mixtures of fluorogenic thrombin substrate and $CaCl_2$ described in the prior art would be expected to precipitate. The Examiner also alleges that the Declaration is insufficient because there are no experiments in which solutions of $CaCl_2$ and ZGGR-AMC are lyophilized separately and rehydrated to form aqueous solutions using varying concentration. In addition, the Examiner alleges that it was known in the prior art that ZGGR-AMC and $CaCl_2$ could be combined to make a clear solution. The Examiner further alleges that the thrombin substrate and $CaCl_2$ are both soluble in water.

5. This Declaration is provided to supply additional information relating to the experiments presented in my previous Declaration and additional data from those sets of experiments. The present Declaration provides additional explanation that fluorogenic thrombin substrates are poorly soluble in water, and that in particular, the fluorogenic substrate in Váradi *et al.* (*J. Thrombosis and Haemostasis* 1:2374-2380, 2003) has very poor solubility in aqueous solutions. Last, this Declaration explains that the combination of thrombin substrate and $CaCl_2$ for the assay described in Váradi *et al.*, typically leads to a precipitate during preparation of the solution, even though the precipitate is not discussed in the publication.

6. In the first set of experiments described in my previous Declaration, small amounts (25 mg) of the fluorescent substrate ZGGR-AMC.HCl in powder form were dissolved in 7.4 mL of HNa-DMSO 10% solution with stirring at room temperature. HNa-DMSO is an organic (i.e., non-aqueous) buffer that contains 10% DMSO, 25 mM HEPES and 175 mM NaCl, pH 7.35). DMSO is required to be present in the buffer because ZGGR-AMC is not soluble in an aqueous buffer. After stirring until the substrate was fully dissolved, a 0.58 ml volume of 1 M $CaCl_2$ was added. A precipitate was observed. This disappeared after stirring. However, after 30 minutes standing at room temperature, the solution was cloudy. After shaking, a distinct, fine

precipitate was noted. After one hour at 37°C, the solution cleared. The absorbance values at 405 nm at these various points were measured. This experiment is described under Experiment 1 on page 1 of the attachment to this Declaration ("the Attachment"). The absorbance values are provided in Table 1. The wells B6 through F6 show the absorbance after the addition of $CaCl_2$. The wells B5 through E5 show the substrate before the addition of $CaCl_2$. Figure 1 of the Attachment shows the different forms of the precipitates during the experiments.

Similar observations were observed in a set of experiment using three samples. The experiment as described above was repeated in triplicate and the absorbance was measured (Experiment 2, page 3 of the Attachment). Table 2 shows the absorbance before the addition of $CaCl_2$. After 0.55 ml of 1 M $CaCl_2$ was added to the dissolved substrate (6.8 ml of substrate), the solution became cloudy. This disappeared after stirring at room temperature. After 30 minutes standing at the bench, two of the three samples became cloudy again. The absorbance is shown in Table 3. Altogether, three of the four samples became cloudy in these experiments using small amounts of the substrate.

7. A second set of experiments was performed using full vials of 250 mg of ZGGR-AMC (Experiments 3 and 4, pages 4 and 5 of the Attachment). In Experiment 3, the substrate was dissolved in 74 ml of HNa-DMSO 10% solution and then 6 ml of 1 M $CaCl_2$ was added to the fully dissolved substrate. A precipitate formed. The solution was warmed at 37° for 15 minutes and the precipitate was dissolved with stirring for 45 minutes at room temperature. The solution then sat at room temperature for 2-3 hours. Visual inspection showed that the solution became cloudy over time. This was confirmed by the absorbance measurements, which are summarized in Table 4. Well C5 is the solution buffer without substrate; C6 is after the substrate was dissolved in the DMSO-buffer solution, C7 is after the addition of $CaCl_2$ and re-dissolving of the cloudy precipitate, D2-D11 are aliquots taken after 2 hours of further storing of the vials at room temperature.

This experiment was repeated (Experiment 4). The solution again became cloudy. Absorbance measurements are shown in Table 5. The time was between 30 to 60 minutes. The development of the precipitate is documented by a series of photographs (Figure 2 of the Attachment).

8. Thus, in the foregoing experiments, there was only one instance in which the solution did not become cloudy out of six instances where the $CaCl_2$ was added to the dissolved substrate. The final desired concentrations (5 mM substrate and 75 mM $CaCl_2$) of the working solutions in these experiments were higher than the concentrations in the final assay conditions used in Váradi *et al.* This higher concentration was required for preparing a working reagent, which has to be added to a reaction mixture of a plasma sample and trigger, to achieve a final concentration of reagents as described in Váradi *et al.*

9. Váradi *et al.* is my work. Váradi *et al.* does not detail the preparation of the thrombin substrate/$CaCl_2$ solution described on page 2375 at the second full paragraph. However, for the experiments performed in Varadi *et al.*, the fluorgenic ZGGR-AMC substrate (250 mg) was first dissolved in 74 ml of a HEPES-NaCl buffer containing 10 % DMSO. Warming and vigorous shaking was required to dissolve the precipitate formed upon the addition of the required amounts (6 ml) of 1 M $CaCl_2$ to the dissolved ZGGR-AMC substrate. This solution had a concentration of 75mM $CaCl_2$ and 5 mM thrombin substrate. Once the precipitate was dissolved, the solution was aliquoted and stored frozen at –20°C. For use, an aliquot was thawed, diluted 5-fold with HEPES-NaCl buffer for use in the reaction mixture, resulting in the final concentration of 15 mM $CaCl_2$ and 1 mM thrombin substrate.

10. Fluorescent substrates are usually not water soluble and after lyophilization an organic solvent such as DMSO is required to re-dissolve the substrate, which is not convenient in a clinical environment. For example, according to the manufacturer, the commercially available ZGGR-AMC substrate has to be dissolved in an organic solution. Lawson *et al.* also describes fluorogenic thrombin substrates, which were first dissolved in DMSO to a stock concentration of 10 mM.

As further evidence of the poor solubility of the fluorescent substrate, we tried to reconstitute ZGGR-AMC (250 mg) in water (74 ml) without DMSO, but the powder could not be fully dissolved. We proceeded with adding $CaCl_2$, even though the substrate was not fully dissolved. A fine precipitate was formed. This could not be solubilized, even after heating to 37°C and stirring for 60 minutes (Experiment 5 and Figure 3).

11. A basic requirement for reagent kits is that the kit be "ready to use" and immediately available, if needed. Therefore a reagent in which a precipitate is likely to form (either immediately or delayed) upon the addition of $CaCl_2$ to the substrate is not an acceptable diagnostic reagent. The current invention provides a lyophilized reagent comprising $CaCl_2$ and a thrombin substrate comprising a fluorescent label. The lyophilized mixture is reconstituted prior to use in an aqueous buffer. The DMSO present in the initial buffer to solubilize the fluorescent thrombin substrate is lost during lyophilization. We discovered that no precipitate forms when the lyophilized mixture is dissolved in the aqueous buffer such that the reagent is "ready to use". Organic solvent is not required. Based on my experience in field working with fluorogenic substrates, this was an unexpected and surprising finding.

12. The Declarant has nothing further to say.

Date: 16.04.2008

By: _____
Peter Turecek, Ph.D.

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